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Degradation of urine samples and its influence on the $^{13}C/^{12}C$ ratios of excreted steroids

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The degradation processes in deficiently stored urine samples are well investigated regarding steroid concentrations and diagnostic ratios, such as the quotient of testosterone divided by epitestosterone. In contrast, nothing is known about the influence on carbon isotope ratios (CIR) by inappropriate storage conditions. In general, it is assumed that degradation, i.e. deconjugation or dehydrogenation, does not change CIR and thus CIR can be used in cases where the steroid profile turns out to be invalid.

Therefore, the CIR of urinary steroids was investigated in different urine samples during the course of degradation over a time period of six months. Several steroids excreted as glucuronides (androsterone (A), etiocholanolone (E), testosterone, pregnanediol (PD) and 5α- and 5β-androstane-3α,17β-diol) or sulfo-conjugated (A, E and androst-5-ene-3β,17β-diol (5EN17b)) were investigated together with their unconjugated correspondents (A, E, PD and 5EN17b) and the main dehydrogenation products (5α- and 5β-androstane-3,17-dion and androst-4-ene-3,17-dion). For this purpose, the exiting methods for CIR determination were extended and validated. In addition, the urinary concentrations of all investigated steroids were monitored.

Particular attention was paid to dehydroepiandrosterone conjugated and unconjugated together with its degradation product 3α,5-cyclo-5α-androstan-6β-ol-17-one as here the strongest influence on CIR was expected.

Keywords: carbon isotope ratio; degradation; doping control; storing conditions

Introduction

For the detection of possible misuse of naturally occurring anabolic androgenic steroids like testosterone (T) anti-doping laboratories use a combination of two techniques. First, the steroid profile is investigated providing relevant information on urinary steroid concentrations and diagnostic ratios like the T/EpiT (ratio of testosterone- to epitestosterone-glucuronide).[1,2] Samples showing suspicious steroid profile parameters are then forwarded to isotope ratio mass spectrometry (IRMS) determination in order to prove either the exogenous or the endogenous origin of urinary steroids.[3–7] Therefore, the $^{13}C/^{12}C$ ratios of endogenous reference compounds (ERC) are compared to the $^{13}C/^{12}C$ ratios of target compounds (TC). $^{13}C/^{12}C$ ratios are expressed as $\delta^{13}C$ values against the international standard Vienna Pee Dee Belemnite (VPDB) based on Equation 1:

$$\delta^{13}C[‰] = \frac{\left(^{13}C/^{12}C\right)_{sample} - \left(^{13}C/^{12}C\right)_{std}}{\left(^{13}C/^{12}C\right)_{std}} \times 1000 \qquad (1)$$

where $^{13}C/^{12}C$ refers to the isotopic composition of sample or standard.[8]

Differences between ERC and TC are expressed as Δ values based on Equation 2:

$$\Delta[‰] = \delta^{13}C_{ERC} - \delta^{13}C_{TC} \qquad (2)$$

For all Δ values of interest, reference ranges were determined and reference limits established.[7,9]

Steroids are excreted into urine as conjugates, namely as glucuronides or as sulfates. These conjugates are stable as long as the specimen is stored cooled or frozen and no microbial contamination takes place. As soon as the urine is not stored properly anymore, different chemical, enzymatic, or microbial degradations can start. First, conjugates are hydrolysed either spontaneously or by enzymatic activity increasing the amount of free steroids. Then these unconjugated steroids can be dehydrogenated at functional groups and the steroid backbone by microbial activity.[10–12] The impact of these degradations on the steroid profile is well investigated,[2,13–15] but no data is published on the possible changes in CIR. It is generally assumed that degradation does not affect CIR. But as this influence might change both the $\delta^{13}C$ and the Δ values, it bears the potential to invalidate the IRMS technique and therefore has to be investigated carefully.

In order to identify any changes in CIR, urine samples were stored at elevated temperatures and investigated regarding both their steroid profile and their CIR of several selected steroid conjugates and the belonging degradation products. Glucuronidated steroids of interest were T, androsterone (A), etiocholanolone (E), pregnanediol (PD), 5α- and 5β-androstane-3α,17β-diol (5a and 5b), and dehydroepiandrosterone (DHEA). Sulfo-conjugated steroids were A, E, DHEA and androst-5-ene-

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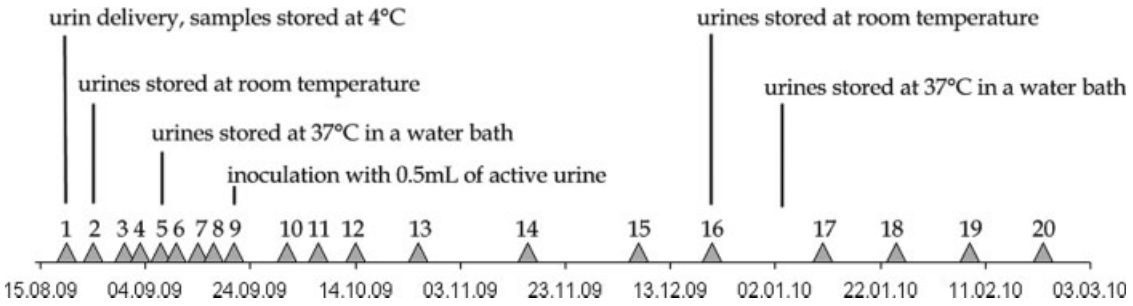

Figure 1. Time schedule of sample treatment, each triangle stands for a sample preparation, further information in the text.

3β,17β-diol (5EN17b) and regarding unconjugated steroids it was possible to determine A, E, PD, DHEA, 5EN17b, 3α,5-cyclo-5α-androstan-6β-ol-17-one (DCM), 5α- and 5β-androstane-3,17-dione (ADN and EDN) and androst-4-ene-3,17-dione (4DN).

For the unconjugated steroids a new high performance liquid chromatography (HPLC) method was developed and carefully validated by means of linear mixing models and repeated analysis of urine specimens fortified with steroid standards.

Special interest was paid on the formation of DCM, as here the largest effects in isotopic fractionation were expected as DCM is formed during the cleavage of DHEA-sulfate under formation of a new carbon-carbon-bond.[16]

Experimental

Chemicals and steroids

Chromabond® C18 cartridges were obtained from Macherey-Nagel (Düren, Germany). Acetone (for gas chromatography), pyridine, acetic anhydride (distilled before use), glacial acetic acid and ethyl acetate were purchased from Merck (Darmstadt, Germany). *Tert.*-butyl methyl ether (TBME, distilled before use) was from KMF Laborchemie (St Augustin, Germany), β-glucuronidase from *Escherichia coli* from Roche Diagnostics GmbH (Mannheim, Germany), and steroid reference material (A, E, PD, DHEA, T, DCM and 5α-androstane-3β-ol (RSTD) as well as 5α-androst-16-en-3α-ol (16EN)) was supplied by Sigma (Steinheim, Germany). 5a, 5b, EDN and androst-5-ene-3β,17α-diol (5EN) were purchased from Steraloids (Newport, RI, USA). β-Estradiol-3,17-diacetate (EST) was from Riedel-de Haen (Seelze, Germany) and 4EN and ADN were from Serva (Heidelberg, Germany). All solvents and reagents were of analytical grade.

Urine samples

Three female and three male volunteers provided single-spot morning urines. All volunteers declared not to use any steroids, prohormones, or dietary supplements. The study was approved by the local ethical committee, and written consent was given by all participants. (Ethical committee of the German Sport University Cologne, Prof. Dr Dr hc Eckhard Meinberg, 12.05.2010).

Storage conditions

All six urine samples were treated in the same way. The time schedule is depicted in Figure 1. Three of the six samples were excluded during the course of the study because of insufficient urine volume (one) and two did not show significant signs of degradation. After storing the samples for four weeks at room temperature, all specimens were placed in a heated water bath at 37 °C to accelerate the degradation processes. Another four weeks later all samples were inoculated with 0.5 mL of active urine already showing strong indications of degradation in order to transfer microorganisms into all urines. In between the study, urines were stored at room temperature again for approximately two weeks as the laboratory was closed during Christmas holidays.

Sample preparation

Analytes have to be efficiently isolated and purified before GC/C/IRMS analysis in order to avoid co-elution of compounds and to keep in readiness the ability to measure differently concentrated urinary steroids in comparable amounts. Both aspects are necessary for valid $^{13}C/^{12}C$ determinations. Therefore, extensive sample preparation followed by HPLC clean-up was employed.

A detailed description of sample preparation was published elsewhere[7,9] and will herein only be described in brief. Ten mL of urine were applied on a conditioned C18 solid-phase extraction cartridge, washed with 2 mL of water and eluted triply with 1 mL of methanol (MeOH). After adding 10 µL of a solution containing 100 µg/mL RSTD in acetone, the dried residue was dissolved in 1 mL of sodium phosphate buffer and extracted with 5 mL of TBME to separate the unconjugated steroids. The aqueous residue was hydrolysed with β-glucuronidase, adjusted to pH 9.6 with 0.5 mL of potassium carbonate buffer and again extracted with 5 mL TBME (glucuronidated steroids). The organic layer was transferred into a conical test tube. The pH of the aqueous residue was adjusted to 5 with 100 to 150 µL of glacial acetic acid followed by another solid phase extraction. The sulfo-conjugated steroids were eluted with MeOH/ethyl acetate, 10 µL of a solution containing 100 µg/mL RSTD in acetone were added and hydrolysed with ethyl acetate/sulfuric acid.[9,10] After adding 0.5 mL of methanolic sodium hydroxide and evaporation to dryness, the residues were reconstituted in 5 mL of water and extracted with 5 mL of TBME (sulfo-conjugated steroids). After centrifugation, the organic layer was transferred into a conical test tube.

Urinary steroid concentrations

A 0.5 mL aliquot of each of the abovementioned organic layers was prepared to determine the amount of different steroids. The glucuronidated steroids were determined according to routine sample preparation procedures.[2] Both dried aliquots of the unconjugated and sulfo-conjugated fraction were acetylated by adding 50 µL of pyridine and 50 µL of acetic anhydride and incubation for 45 min at 70 °C. After evaporation to dryness the samples were transferred into auto-sampler vials, evaporated, redissolved

in 10 µL of TBME and forwarded to gas chromatography/mass spectrometry (GC/MS) determination.

For this purpose a GC Agilent 6890 coupled to a mass selective detector MSD Agilent 5973 was used. The GC system was equipped with a Macherey & Nagel OPTIMA δ3 column (length 20 m, i.d. 0.25 mm, film thickness 0.25 µm). The injections were performed splitless at 300 °C. The initial oven temperature of 60 °C was held for 1.5 min, increased at 40 °C/min to 240 °C, followed by a ramp at 2 °C/min to 260 °C, and 40 °C/min to the final temperature of 300 °C. A constant flow of 1.2 mL/min with helium as carrier gas was used. The MSD acquired data in single ion monitoring mode. The following ions were used for quantification and identification, respectively: RSTD (243/258), DCM (270/121), E and A (272/244), EDN and ADN (288/244), 4DN (286/244), PD (284/269), DHEA (270/255) and 5EN17b (314/286) [m/z].

HPLC clean-up

The 4.5 mL TBME residue of each fraction was evaporated to dryness, redissolved in 200 µL of acetone, transferred into HPLC auto-sampler vials and evaporated. The clean-up methods for steroids excreted glucuronidated and sulfo-conjugated have already been published elsewhere.[7,9] For the unconjugated compounds the existing method was adopted as described herein.

The HPLC clean-up was performed on an Agilent 1100 HPLC system (Waldbronn, Germany) equipped with a Merck analytical column (LiChrospher® 100 RP^{18}, 250 × 4 mm i.d., 5 µm particle size).

For the first run, a linear gradient increasing from 30/70 acetonitrile/water to 100% acetonitrile in 25 min was used. After 5 min at 100% acetonitrile, the column was reequilibrated for 5 min; 50 µL injection volume (acetonitrile/water 60/40) and flow rate 1 mL/min were used. Before each batch of samples, a standard solution containing approximately 100 µg/mL of 5EN17b, 4DN, DHEA, DCM, E, A, PD and EST each was injected twice to determine the retention times for fraction collection. The automatic fraction collector Foxy 200 from Isco (Lincoln, NE, USA) was programmed to prepare four fractions as illustrated in Figure 2A.

Fraction II (DCM) was evaporated and directly forwarded to IRMS measurement, to fraction IV 10 µL of a solution containing 50 µg/mL 5EN was added, evaporated to dryness, acetylated and then forwarded to IRMS.

Fraction III was divided into two parts and evaporated. One half was directly forwarded to IRMS for E and A determinations, the second half was acetylated after addition and evaporation of 10 µL of a solution containing 50 µg/mL 16EN and another HPLC run was performed to separate E and A from ADN and EDN (Figure 2B). The HPLC conditions were maintained as for the first run. The time frames for fraction collection were determined with a standard solution containing approx. 100 µg/mL E, A, AND, EDN and 16EN as acetates.

To Fraction I 10 µL of a solution containing 50 µg/mL RSTD was added, evaporated and acetylated. For the second HPLC clean-up, a different gradient was used. From 60/40 acetonitrile/water a linear increase to 100% acetonitrile was accomplished in 33 min and maintained for 5 min. Subsequently, the column was re-equilibrated for 5 min. The flow rate was set to 1 mL/min, injection volume was again 50 µL. Steroids were collected as depicted in Figure 2C, retention times were estimated by two subsequent runs of a standard containing approx. 100 µg/mL 4DN, DHEA, EST, 5EN17b and RSTD as acetates. 5EN17bAc and RSTDAc were combined and all fractions were evaporated to dryness under a stream of nitrogen and then forwarded to IRMS measurements.

GC/C/IRMS measurements

All samples were measured on an Agilent 6890 Gas Chromatograph (Waldbronn, Germany) coupled to a Delta plus XP gas isotope ratio mass spectrometer (ThermoElectron, Bremen, Germany) via a modified GC combustion interface (GCC III, ThermoElectron).[17] The GC system was equipped with the same column as mentioned above. Injection was performed with a Gerstel (Mühlheim an der Ruhr, Germany) KAS unit at 50 °C in solvent vent mode with a helium flow of 100 mL/min. Injection volumes ranged from 2 to 5 µL of TBME. The initial temperature was maintained for 3 min and increased at 40 °C/min up to 260 °C, then at 2 °C/min to 280 °C and then at 40 °C/min up to 295 °C and kept for 3 min. Carrier gas was purified He (purity grade 5.0) with a constant flow of 2.4 mL/min. The combustion furnace was operated at 960 °C.

Correction for the acetate moiety

All determined values were corrected for the influence of the acetate moiety as described in literature.[7,18]

Method validation

The developed method for unconjugated steroids was validated by means of a linear mixing model for DCM[7,19] using Equation 3:

$$\delta^{13}C_m = \left(\delta^{13}C_e - \delta^{13}C_a\right)\frac{c_e}{c_m} + \delta^{13}C_a \qquad (3)$$

with c_x = corresponding concentration and $\delta^{13}C_x$ = corresponding $\delta^{13}C$ value; subscript m stands for mixture, e for endogenous and a for added standard.

A comparison with the linear equation ($y = a \times x + b$) shows that the corresponding equation of the resulting line of best fit represents the difference in $\delta^{13}C$ values between the endogenous steroid and the added standard ($\delta^{13}C_e - \delta^{13}C_a$) as its slope. The absolute $\delta^{13}C$ value of the standard is represented by the intercept on the y-axis ($b = \delta^{13}C_a$).

For the other novel steroids (EDN, ADN and 4DN) by repeated preparations of blank urines fortified with steroid standards. The obtained $\delta^{13}C$ values were compared to the $\delta^{13}C$ values of the added standards.

Results and Discussion

Method validation

The approach of linear mixing models was chosen to study the method's validity for DCM. The results for the slope a (10.9 ± 0.29‰) and the intercept b (−32.3 ± 0.14‰) perfectly reflect the values of the used standard of DCM (−32.4 ± 0.36‰) and the value of the endogenous DCM (−21.5‰). The validation results are comparable to those found for other investigated steroids and prove the validity of the method.[7,9,19] The results obtained for urines fortified with standards are listed in Table 1. No significant differences (Student's *t*-test) were found for all investigated steroids. According to this finding, no isotopic fractionation was taking place during sample preparation which might have influenced and invalidated the IRMS measurements.

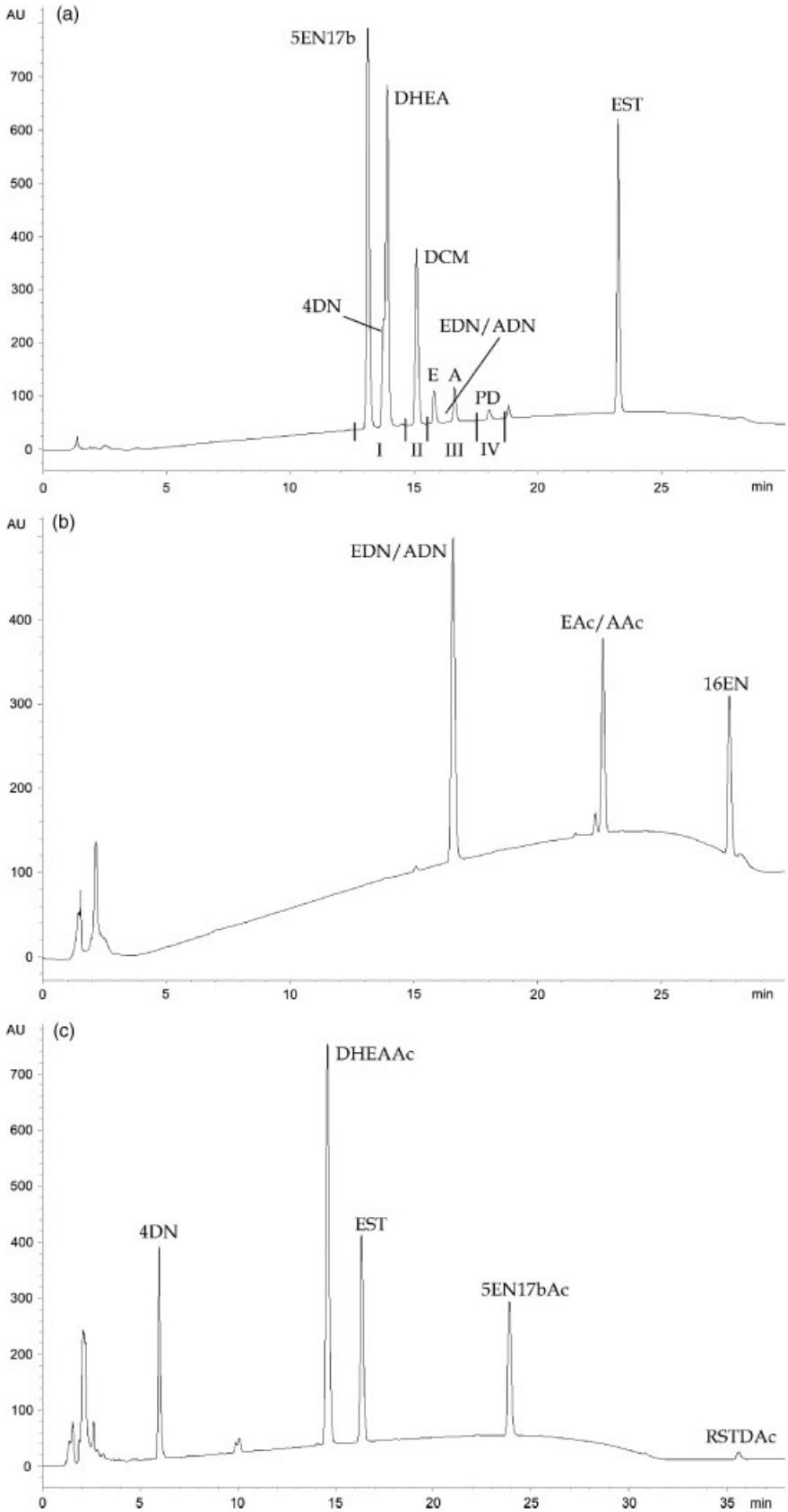

Figure 2. HPLC chromatograms of standards recorded at a wavelength of 192 nm. 2a) HPLC chromatogram of a standard containing 5EN17b, 4DN, DHEA, DCM, E, A, PD and EST. 2b) HPLC chromatogram of a standard containing EDN, ADN, EAc, AAc and 16EN. 2c) HPLC chromatogram of a standard containing 4DN, DHEAAc, 5EN17bAc, RSTDAc and EST.

Table 1. $\delta^{13}C_{VPDB}$ values [‰] of standards and fortified blank urines processed with the described method. SD stands for standard deviation

steroid	standard mean (n = 3)	SD	sample mean (n = 6)	SD
ADN	−31.1	0.14	−31.2	0.13
EDN	−28.7	0.15	−28.7	0.15
4DN	−29.2	0.08	−29.0	0.26

Urinary steroid concentrations

Over the course of time, the urinary steroid concentrations changed as expected. In Figure 3, the concentrations of E excreted as glucuronide (E_G), excreted as sulfo-conjugate (E_S) and found unconjugated (E_F) are depicted together with the concentrations of the oxidation product EDN. The E_G representing the largest pool in urine only declines very slowly. The smaller pool of E_S shows a more pronounced but still moderate diminution. As expected, unconjugated E_F rises shortly after the beginning of the study and starts to decline when the formation of EDN becomes significant.

In general, the described trend was observable for all steroids but some showed a little different behaviour. The degradation product of T, 4DN, emerged earlier than ADN and EDN and was found at higher concentrations in the beginning, and then especially EDN became more abundant. 5EN17b came out to be quite stable. In the beginning, no unconjugated 5EN17b was found. It took more than eight weeks until the first free 5EN17b was found and then it raised slowly and constantly until the end of the study.

CIR of urinary steroids

The results of CIR are presented in different sections. First the glucuronidated steroids will be discussed, than the sulfated ones, followed by unconjugated steroids and at the end the degradation of DHEA will be investigated in detail. In each section only one specimen will be shown exemplarily but the described trends were found in all three urines. To ensure that the found trends were not due to a long term drift of the IRMS, this was monitored with repeated injections of a standard mixture and repeated processing of a blank urine, covering both changes in sample preparation and IRMS determinations.

CIR of glucuronidated steroids

In order to differentiate between an endogenous or exogenous source of urinary steroids in the context of doping control analysis, usually the glucuronides of E, A, T, 5a and 5b are investigated as TC. The most common and reliable ERC is PD.[3–7] Therefore, these steroids were chosen from the pool of glucuronidated steroids and investigated herein. The results obtained from one male urine sample are depicted in Figure 4. This specimen was chosen as the amount of lower concentrated glucuronidated steroids like T and 5a only in this urine was sufficient for IRMS determination until the end of the study.

Within the first three weeks no significant change in CIR was detected for any of these steroids. All show a trend to more depleted values (approximately 0.5‰). But as this happens for both the TC and the ERC, no change in Δ values is visible. In the following weeks the trend to more depleted values continuous but still no significant change in Δ values was found. Around 14 October, this trend turns back for PD, E, A and 5b while T still tends to more depleted values. For PD and E the values became even more enriched than at the beginning of the study. This strange behaviour came along with increasing amounts of oxidation products (EDN, ADN and 4DN) found in urine (compare Figure 3). A detailed description and possible explanation of the processes of isotopic fractionation will be given further on in the text.

Due to the different response of the ERC (PD) and T and 5a, now a significant increase in Δ values for these steroids was found (from 1.7 ‰ to 3.8 ‰ for T and from 2.6 ‰ to 3.5 ‰ for 5a). These Δ values are still below the established reference limits for this IRMS method (PD-T = 4.4 ‰ and PD-5a = 3.7 ‰)[7] but obviously for both steroids the chance for a false positive testing is feasible. This effect has to be taken into account for doping control samples showing strong indications of degradation.

CIR of sulfated steroids

In Figure 4C the results obtained for the sulfated steroids from the same male athlete are depicted. In the beginning, E and A

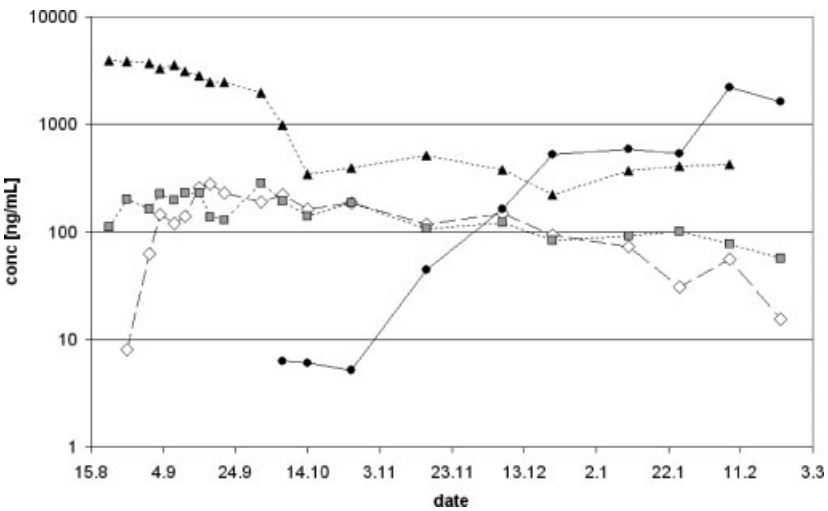

Figure 3. Changes in urinary concentrations of E and EDN exemplarily in one specimen. Black triangles stand for E_G, grey squared for E_S, open diamonds for E_F and black circles for EDN.

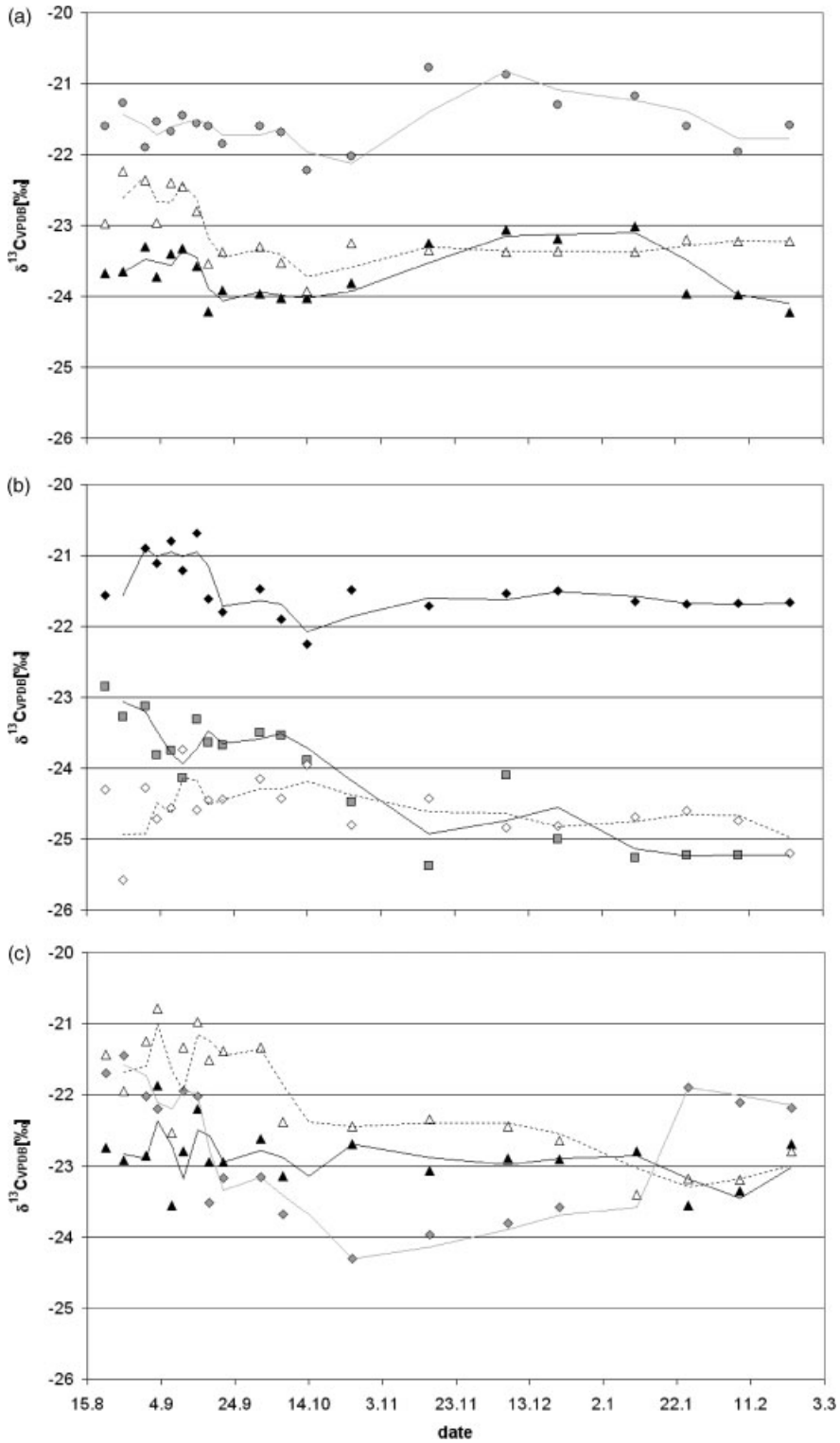

Figure 4. Changes in CIR over time. 4a) Glucuronidated steroids, grey circles stand for PD, open triangles for A and black triangles for E. 4b) Glucuronidated steroids, black diamonds stand for 5b, grey squares for T and open diamonds for 5a. 4c) Sulfated steroids, black triangles stand for E, open triangles for A and grey diamonds for 5EN17b. The trend lines demonstrate the moving average (k = 2).

did not show any change in CIR, after 6 weeks A starts to show a depletion of 1 ‰ which increases over time up to 2 ‰. E_S was not influenced at all. However, 5EN17b showed a significant depletion even at the beginning and was depleted by approx. 2 ‰ in the middle of the study. Similar to some of the glucuronidated steroids, the trend to more depleted values turns back and 5EN17b became a little bit enriched after week 10 of the study and at the end the values were similar to the starting values. The trend for 5EN17b was not comparable in all investigated specimens. In one female specimen the depletion was as high as 4 ‰ while in the other

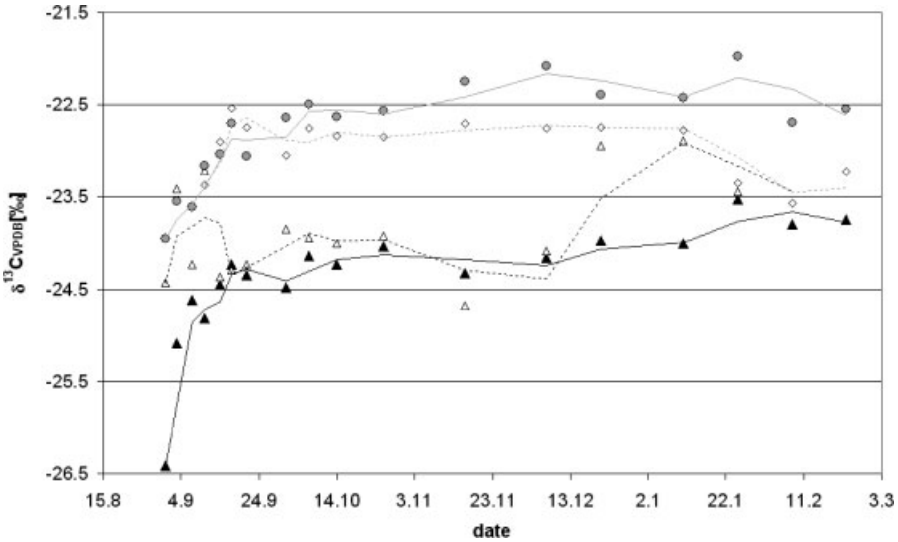

Figure 5. Changes in CIR of unconjugated steroids in the course of time. Grey circles stand for PD, open diamonds for 5EN17b, open triangles for A and black triangles for E. The trend lines demonstrate the moving average (k = 2).

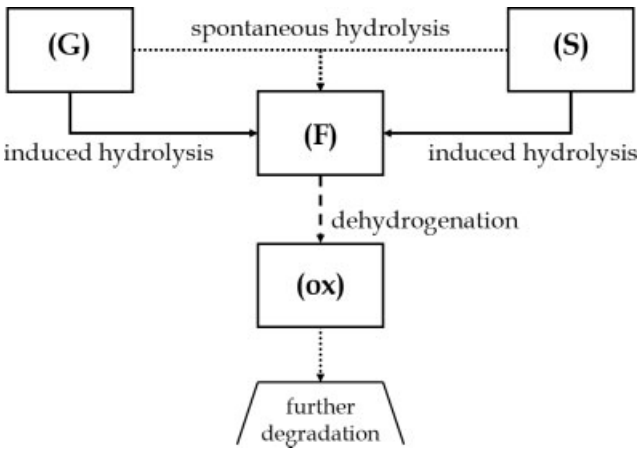

Figure 6. Reaction network for the degradation of steroid-conjugates in urine. Further information in the text.

female urine an enrichment of 5EN17b of more than 2 ‰ was found.

CIR of unconjugated steroids

Interestingly, the 5EN17b in the 'free' fraction did not mirror the changes in the sulfated fraction in any of the specimens under investigation. Throughout the whole study it scattered around its mean value (−22.8‰ for example in the male specimen as can be seen in Figure 5). A_F did not follow a clear trend in any of the investigated specimens and just scattered around its mean value, too. The 5β-steroids E and PD however showed significant depletion at the beginning of the study of more than 2‰. In one female specimen the found depletion accounted for 3.5‰. After *c.* 6 weeks the values stabilized and afterwards only showed slightly enrichment over time.

Interpretation of found CIR

In order to explain the isotopic fraction taking place during degradation, the reaction network of the different processes has to be taken into account (Figure 6). Unfortunately, the network is complicated regarding isotopic fractionation as the following basic conditions have to be considered:

1) The pool of glucuronidated steroids (G) is much larger than the pool of sulfo-conjugates (S). Thus, effects coming along with the cleavage of sulfates might be superposed by effects of hydrolysis of glucuronides.
2) There are two possible reactions yielding unconjugated steroids which fill up the pool of 'free' steroids (F). Both the spontaneous and the induced hydrolysis might be accompanied by isotopic fractionation and both fractionation factors do not have to be equal. But due to the design of the study it was impossible to distinguish which reaction contributes to the ascertained effects in which way.
3) The F-pool is just an intermediate and its CIR is not only affected by the flux into the pool but also by the efflux to the pool of oxidized steroids (OX). An accompanied isotopic fractionation for dehydrogenation will also influence the CIR of this pool.[20]
4) The same can be stated for the OX-pool, but as the amount of steroid in this pool increased until the end of the study, further degradation of steroids can be neglected.

In Figure 7, the values obtained for E_G, E_S, E_F and EDN of one male specimen are summarized exemplarily. Obviously, one or both of the hydrolysis reactions is accompanied by strong isotopic fractionation. This finding was not expected at all as during the de-conjugation of steroid conjugates no carbon atom of the steroidal backbone is involved.[21–25] For both the glucuronides and the sulfates, the bond-fission is expected between the conjugate residue and the oxygen atom and not between the steroid backbone and the oxygen. Only for A_S, a cleavage of the C-O bond is described in literature.[26] But this only occurs under treatment of A_S with boiling hydrochloric acid and thus seems not to be comparable to the reaction conditions in this study. As mentioned above, the efflux from the F-pool to the OX-pool can influence the CIR of unconjugated steroids, but especially at the beginning no oxidation products could be detected in the specimens. So the hydrolysis itself must come along with isotopic depletion. This

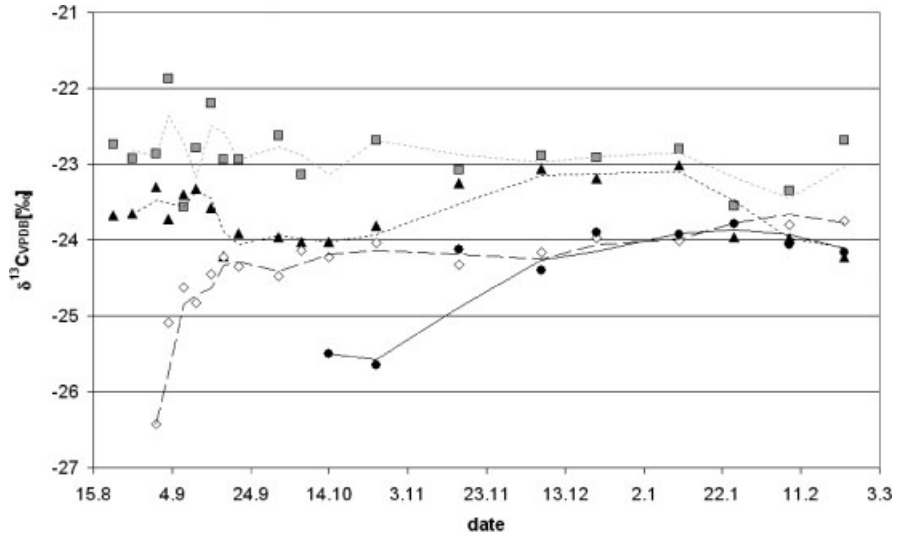

Figure 7. Changes in CIR of etiocholanolone found in one male urine sulfated (grey squares), glucuronidated (black triangles), unconjugated (open diamonds) and EDN (black circles). The trend lines demonstrate the moving average (k = 2).

Table 2. Mean $\delta^{13}C_{VPDB}$ values [‰] of the last three preparations of one male urine specimen. SD stands for standard deviation

steroid	(S)	SD	(G)	SD	(F)/(ox)	SD
E	−23.20	0.45	−24.05	0.14	−23.69	0.14
BDN					−24.00	0.19
A	−23.06	0.22	−23.22	0.02	−23.09	0.30
ADN					−23.65	0.38
T			−25.23	0.03		
4DN					−25.13	0.12

should result in isotopic enrichment of the conjugated pools and as can be seen in Figure 7, E_G shows a very slightly enrichment at the beginning in coincidence with the occurrence of E_F. This trend is visible in all three investigated specimens. As the G-pool is much larger than the F-pool, the influence on CIR was expected to be very small. In the S-pool no similar trend was visible. These results suggest a strong isotopic fractionation during hydrolysis of a subset of glucuronidated steroids, but further studies on this topic should be conducted.

The impact of this fractionation on 5β-steroids (PD, E) is much larger than on the other ones. So maybe this can be attributed to an enzymatic cleavage with preference on 5β-isomers only taking place at the beginning. Shortly after, another enzymatic reaction started and superposed that one, not being accompanied by such a strong isotopic fractionation. For doping control purposes the fractionation has not to be taken into account as the effect on the glucuronidated steroids is negligible.

With emerging of the oxidation product EDN, again the values of E_G are strongly influenced while neither E_S nor E_F shows a significant response. Here it seems to be a direct conversion of E_G to EDN with isotopic depletion of the oxidation product as both pools show parallel changes in their $\delta^{13}C$ values. Again, and in accordance with literature,[12,14] the 5β-steroids were stronger influenced. At the end of the study, when the whole reaction network was assumed to be in equilibrium, a direct comparison of the CIR reveals, that the net isotopic fractionation between the different pools is quite small and therefore in accordance with the expected values prior to this study. The results of the last three sample preparations are summarized in Table 2. As already reported in literature, steroid sulfates show more enriched values than glucuronides.[9] Despite the strong isotopic fractionation at the beginning of the study, at the end in equilibrium the difference between unconjugated and glucuronidated steroids was found negligible. The same holds true for comparison of the dehydrogenated and the glucuronidated steroids. Even T, showing the strongest depletion in its G-pool due to its relative low cocentration ended up with stable CIR not showing any difference between T_G and 4DN.

Due to the, regarding isotopic fractionation, complicated reaction network, it is not possible to identify the different sources of fractionation and their impact on CIR unambigously. Further studies focussing on single aspects of the reaction network should be carried out. By that time a model for the fractionation factors and CIR of the different pools can be calculated. In the context of doping control, it has to be taken into consideration that urine samples showing degradation might have influenced CIR of investigated steroids. Especially if oxidation products found in urine specimens, CIR should be handled with care as is already usual practise for steroid profile parameters.

CIR of DHEA and DCM

The conversion of DHEA_S to DCM was expected to be accompanied by strong isotopic fractionation due to the bond formation between C3 and C5 in the steroidal A-ring in addition to the increased tension in this ring. A concerted reaction mechanism is assumed, directly yielding DCM under cleavage of the sulfate moiety and the steroidal backbone.[16] The results obtained in this study (Figure 8) do not support the assumption of a concerted reaction but encourage an ionic transition state.[27–29]

While DHEA_G was not involved in the formation of DCM as it did not show any significant change in CIR over time, DHEA_S showed strong enrichment in its $\delta^{13}C$ values from the beginning on. Starting with values around −21‰, it ended up with values of −11‰ before the amount of DHEA_S was found too low for CIR determinations. Both the values of DHEA_F and DCM showed the corresponding $\delta^{13}C$ values with −26‰ and −25‰ at the

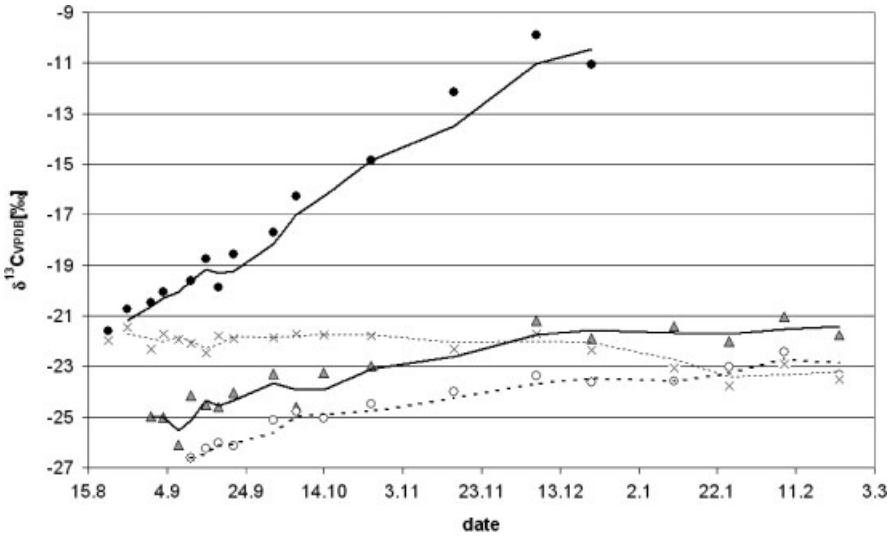

Figure 8. Changes in CIR of DHEA and DCM found in one female urine. Black circles represent DHEA_S, crosses DHEA_G, grey triangles DCM and open circles DHEA_F. The trend lines demonstrate the moving average (k = 2).

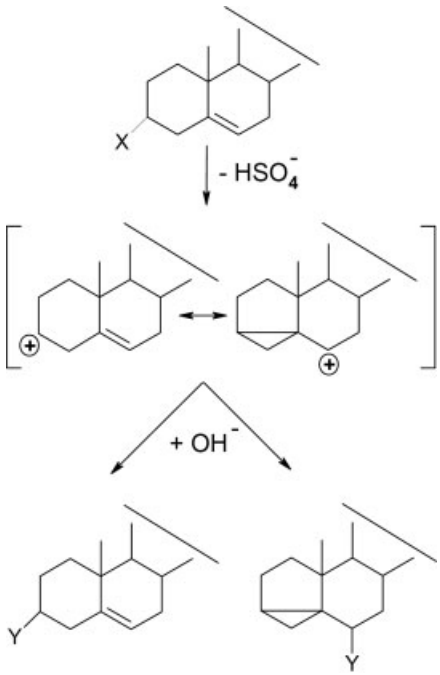

Figure 9. Proposed reaction scheme for the simultaneous formation of DHEA_F and DCM. Adopted from Winstein and Adams.27 Further information in the text.

beginning, then a constant rise to and steady values of −23‰ and −22‰, respectively, at the end when the DHEA_S pool became negligible. The found values were in perfect agreement with the theory of isotopic fractionation and its influence on CIR.[20] As DHEA_F and DCM showed a parallel influence on their $\delta^{13}C$ values, a concerted reaction from DHEA_S to DCM is not likely as both products CIR should have been influenced in different ways. DHEA_F should have been influenced like the other unconjugated steroids only at the beginning of the study while DCM and DHEA_S might correlate as demonstrated in Figure 8. The parallel influence of both steroids indicates the existence of an ionic transition state depicted in Figure 9. The initial step in the formation of both DCM and DHEA_F is the loss of HSO_4^- yielding the transition molecule with a delocalized positive charge from C3 to C6.[28] The intermediate than reacts with the nucleophilic agent OH^- either at position 3 resulting in unconjugated DHEA or at C6 resulting in DCM. The rate limiting step accompanied by major isotopic fractionation must be the initial cleavage of the sulfate moiety. The following nucleophilic saturation prefers C6 and might introduce another source for isotopic fractionation resulting in Δ(DCM-DHEA_D) = 1‰. This preference of C6 addition has already been described in literature [28,29] and was reflected by the found urinary concentrations in this study with a 3- to 5-fold amount of DCM over DHEA_F.

According to these results, DCM should not be used as a TC in doping control analysis as it might be affected by strong isotopic fractionation and does not necessarily reflect an endogenous $\delta^{13}C$ value even in urine samples not showing any signs of degradation. These finding does not affect the usefulness of DCM for steroid profiling.

Furthermore, the isotopic fractionation described for DHEA_S might bear an explanation for the found CIR of 5EN17b not being in line with the found values of other investigated steroids. A formation of a 3,5-cyclo metabolite is likely for 5EN17b_S, too. Unfortunately, this possible metabolite is not described in the literature and was not under investigation herein. Especially in the female sample presented in this paragraph with its high amount of DCM, the 5EN17b_S showed a parallel enrichment to DHEA_S at the beginning of the study. This might be an indicator for a parallel reaction of the 5EN17b but further studies on this possible formation of 3,5-cyclo metabolites are necessary to evaluate these findings.

Conclusion

The influence on CIR of different urinary steroids and steroid-conjugates during degradation was investigated. Regarding glucuronidated steroids which are used in doping control analysis, no significant influence on CIR during the first weeks of the study could be detected. This changed with the emergence of dehydrogenation products 4DN, ADN and EDN and therefore

suggested careful interpretation of CIR results in samples showing these strong indications of degradation. The same applied for steroids excreted as sulfates. Especially at the beginning, unconjugated steroids showed strongly depleted $\delta^{13}C$ values and therefore shall not be utilised in doping control analysis. The reasons for this strong fractionation could not be identified unambiguously within this study and further research on the deconjugation of steroid glucuronides seems advisable.

The results obtained for DCM and DHEA supported the theory of a reaction mechanism including an ionic intermediate rather than a concerted reaction. In the context of doping control analysis, the CIR of DCM, DHEA_S and maybe 5EN17b_S should not be taken into consideration due to the strong isotopic fractionation coming along with the cleavage of the sulfate moiety.

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